

# DRUG DISCOVERY INTERFACE

## Extreme Sensitivity of Botulinum Neurotoxin Domains Towards Mild Agitation

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**ABSTRACT:** Botulinum neurotoxins (BoNTs) and their fragments are targets of therapeutic developments and are increasingly used as therapeutic, prophylactic, and research reagents. However, published data on their properties vary widely. In order to gain a better understanding of these variations, we initiated a systematic investigation of the stability parameters of catalytic light chains (Lc) as well as of cell surface binding domains (Hc) of the neurotoxin. When followed by CD spectroscopy, we noticed that the recombinant light chains of serotypes A (LcA), B, D, E, and G rapidly lost their secondary structures by mild stirring. Denaturation of LcA increased with stirring speed and temperature resulting in a catalytically inactive precipitate. Reducing agents or an anaerobic environment were ineffective in the denaturation. Under identical conditions, bovine serum albumin, ovalbumin, carboxypeptidase B, and of thermolysin, a structural and functional analogue of LcA, remained unchanged. Hc domains of serotype A, B, C, E, and F were also denatured by mild stirring. Adding the nonionic detergent Tween-20 to LcA completely prevented the denaturation. We speculate that the BoNT domains undergo surface denaturation due to rapid exposure of hydrophobic residues by mechanical agitation. This study has important implications for handling BoNT proteins used in therapeutic development. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 98:3302–3311, 2009

**Keywords:** circular dichroism; solubility; proteins; protein aggregation; surfactants; thermodynamics; enzyme; botulinum neurotoxin; precipitation; inactivation

### INTRODUCTION

Understanding and monitoring the stability of proteins are important factors in their therapeutic

applications. Botulinum neurotoxins (BoNTs) are most lethal of all toxins.<sup>1,2</sup> The neurotoxins and their nontoxic fragments are also finding increasing application as therapeutic and prophylactic

Abbreviations: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin serotype A; LcA, LcB, LcC, etc., light chain of BoNT serotypes A, B, C, etc.; HcA, HcB, HcC, etc., heavy chain C-terminal domain of BoNT serotypes A, B, C, etc.; H<sub>n</sub>, N-terminal domain of the heavy chain; H<sub>c</sub>, C-terminal domain of the heavy chain; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; SNARE, soluble NSF attachment protein receptor; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CD, circular dichroism.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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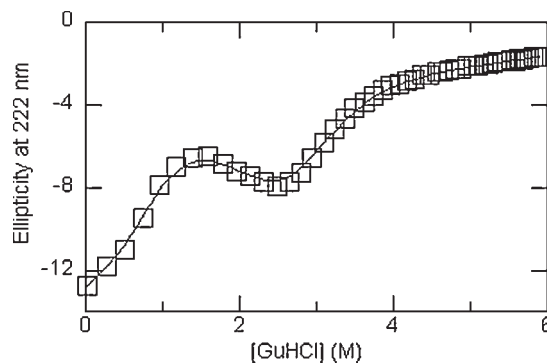
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agents,<sup>3-9</sup> and are targets of drug development.<sup>10-13</sup> These neurotoxins are initially expressed by strains of *Clostridium botulinum* as 150-kDa single polypeptides along with other accessory proteins yielding a 900 kDa complex. The latter BoNT complex was recently shown to be more stable *in vitro* than the free 150 kDa BoNT.<sup>14</sup> Posttranslational cleavage of the 150 kDa protein by an endogenous trypsin-like protease generates a 50-kDa N-terminal light chain (Lc or LC) and a 100-kDa C-terminal heavy chain (HC) that remain connected by a disulfide bond. The 100-kDa HC can be further proteolyzed into a 50-kDa N-terminal membrane-spanning domain (H<sub>n</sub>) and a 50-kDa C-terminal receptor-binding domain (H<sub>c</sub>). The LC possesses the toxic, zinc endopeptidase catalytic domain, but in the absence of HC, it is nontoxic. The nontoxic nature of LC and Hc domains make them ideal tools for drug development and as vaccine candidates.

The catalytic activity of the recombinant LC of serotype A (LcA) has been reported to vary by more than sevenfold using its full-length SNAP-25 substrate<sup>15,16</sup> and more than fourfold using a 17-mer SNAP-25 peptide as a substrate Ahmed 2006, Unpublished].<sup>17,18</sup> Similarly, the reported midpoint of thermal denaturation of LcA also varies considerably,<sup>16,17,19,20</sup> and its protective immunity varied from no protection to 90% survival of mice challenged with the whole BoNT toxin.<sup>21</sup> In addition, differences in the solubility of different batches of LcA were observed in various labs Ahmed 2006, Unpublished].<sup>22</sup> These differences were usually attributed to differences in the LC purification, batch, or experimental procedures. To overcome the solubility problems, various c-terminal truncated LcA were constructed, and those representing residues 1-425 and more were shown to display high solubility and retain optimal activity.<sup>22</sup> Yet, various labs have differing experience on their activity.

Because of their therapeutic importance, we initiated a study to determine both catalytic and physical stability of LCs of various serotypes under a standard set of experimental conditions. While determining the free energy of unfolding by CD spectroscopy using an automatic titrator containing guanidine hydrochloride and a built-in magnet for stirring the LC in a cuvette, we observed a very unusual unfolding behavior (Fig. 1). With increasing concentration of guanidine hydrochloride, the ellipticity of LcB first increased, then decreased before increasing again to equilibrium (Fig. 1). After repetition of the



**Figure 1.** Guanidine hydrochloride denaturation curve of LcB. The protein solution (20  $\mu\text{g/mL}$ ) was continuously mixed with guanidine hydrochloride from an automatic titrator by a built-in magnetic stirrer at 20°C, and the ellipticity was monitored. Note the absence of a baseline at low guanidine hydrochloride concentration and of a flat region between 1.5 and 2.7 M guanidine hydrochloride concentration. The decrease in ellipticity at the latter guanidine hydrochloride concentration is highly unusual.

experiment under various conditions and careful analysis of the results, we discovered that the observed unusual unfolding behavior was related to mechanical stirring of the LC solution by the magnetic stirrer. Such mechanical stirring is an integral part of automatic titration experiments but has never been reported before as causing protein precipitation. In this paper we demonstrate that, unlike the standard proteins of BSA, ovalbumin, and thermolysin, the LCs of serotype A, B, D, G, and E are highly susceptible to denaturation, and mild mechanical agitation is enough for their precipitation by aggregation. We also found that the Hc domains of serotype A, B, C, E, and F were similarly denatured by stirring. The denaturation was however completely prevented by adding Tween-20 to a solution of LcA. Our demonstration of this phenomenon may yield a clue to the discrepant results obtained and reported on BoNT domains by laboratories, including ours.

## EXPERIMENTAL PROCEDURES

### BoNT/A LC, Chemicals, Buffers, and Reagents

The 449-residue recombinant BoNT/A LC (LcA) with an extra valine residue at position 2<sup>18</sup> was expressed and purified as described.<sup>17</sup> The homo-

geneous preparation was stored at  $-20^{\circ}\text{C}$  in 50 mM Na-phosphate, pH 6.5 containing 150–250 mM NaCl and 2 mM EDTA. Recombinant LcB was purified as published,<sup>23</sup> those of LcD and LcG will be published elsewhere. Recombinant LcE was purchased from Bibitech (Dartmouth, MA). Recombinant Hc proteins were purified in Dr. Smith's laboratory. Before use, each 1 mL of LC was incubated with 2.5 mM zinc chloride on ice for 30 min followed by buffer exchange in 10 mM sodium phosphate buffer, pH 7.4 by passing through a PD-10 column. Unless otherwise mentioned, all other proteins were diluted in 10 mM Na-phosphate, pH 7.4 to a final concentration of 20  $\mu\text{g/mL}$ . Anthrax lethal factor (1 mg/mL solution in 5 mM HEPES, 50 mM NaCl, pH 7.5, List Biological Laboratories, Inc., Campbell, CA) was a gift from Dr Sina Bavari (USAMRIID). Carboxypeptidase B (Worthington Biochemical Corp., Lakewood, NJ) was obtained as a 22.3-mg/mL solution (5 mg) in 100 mM sodium chloride. Bovine serum albumin (BSA, Sigma, San Diego CA), ovalbumin (Sigma), and alcohol dehydrogenase (Worthington Biochemical Corp.) were purchased as lyophilized powder and reconstituted as 3 mg/mL solutions in 10 mM phosphate buffer, pH 7.4. Thermolysin (Calbiochem, St. Louis, MO), obtained as a lyophilized powder (61.6% protein, 12.4% sodium acetate, 23.8% calcium acetate), was reconstituted in the company recommended buffer (42% glycerol, 0.005% Triton X-100, 10 mM sodium phosphate, pH 8.0) to give a final protein concentration of 4.96 mg/mL.

### Enzymatic Activity Assays

The enzymatic assay was based on HPLC separation and measurement of the cleaved products from a 17-residue C-terminal peptide corresponding to residues 187–203 of SNAP-25.<sup>18,24</sup> A master reaction mixture lacking the LC was made and aliquots were stored at  $-20^{\circ}\text{C}$ . At the time of assay, an aliquot of the master mix was thawed and 25  $\mu\text{L}$  was added to 5  $\mu\text{L}$  of the LC (see above) to initiate the enzymatic reaction. Components and final concentration in this 30- $\mu\text{L}$  reaction mixture was 0.9 mM substrate peptide, 0.5  $\mu\text{M}$  LC, and 50 mM Na-HEPES, pH 7.4 with or without 0.25 mM  $\text{ZnCl}_2$ , and 5 mM dithiothreitol (DTT). After 5 min of incubation at  $37^{\circ}\text{C}$ , the reaction was stopped by acidifying with 90  $\mu\text{L}$  of 1% trifluoroacetate (or acetic) TFA, and 100  $\mu\text{L}$  of this mixture was analyzed by HPLC as described.<sup>18</sup>

### UV-Visible Absorption, Circular Dichroism, and Fluorescence Measurements

To determine protein concentration and to assess purity, UV-visible absorption spectra were recorded at  $22^{\circ}\text{C}$  with a Hewlett–Packard 8452 diode array spectrophotometer. LC concentration was determined using  $A^{0.1\%}$  (1 cm light path) value of 1.0 at 278 nm<sup>25</sup> or by BCA assay (Pierce) with BSA as standard. Both methods give the same result.

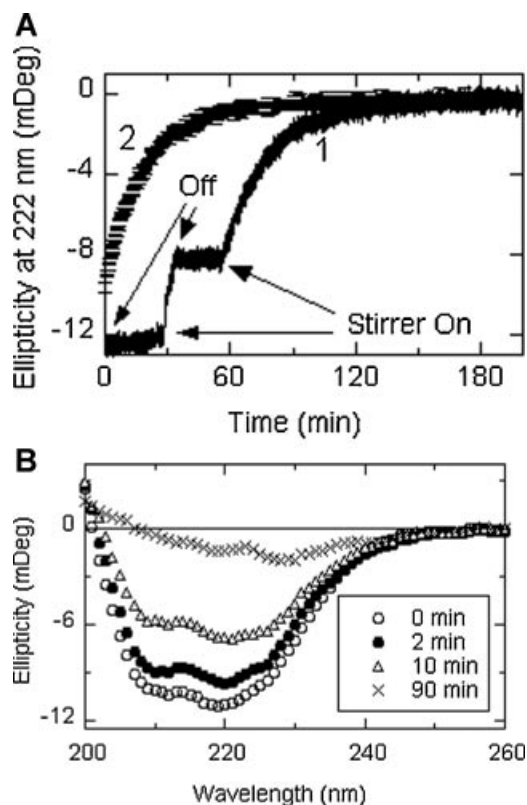
Circular dichroism spectra were recorded at  $20^{\circ}\text{C}$  unless otherwise indicated, with a Jasco 718 spectropolarimeter equipped with a Peltier-controlled sample holder, a magnetic stirrer, and a Jasco PFD-425S temperature controller. Protein samples (usually 2.7 mL, 20  $\mu\text{g/mL}$ ) were taken in 3-mL quartz cuvettes of 1-cm path length, and were under an inert nitrogen ( $\text{N}_2$ ) atmosphere. In some experiments, as indicated, nitrogen was replaced by argon. CD Spectra were recorded at  $20^{\circ}\text{C}$  with a scan speed of 50 nm/min and response time of 8 s. In all measurements, a buffer blank was recorded separately and subtracted from sample recordings. CD time-course measurements were recorded at 222 nm. Sample was agitated normally at a speed of 1200 rpm either with a “Micro-Flea Magnetic Stir Bar” (Fisher brand; 8 mm  $\times$  2 mm) or a “Star Head Stir Bar” (Nalgene; 10 mm  $\times$  8 mm) placed in the cuvette held in a Peltier controlled thermostated chamber. This stirring did not produce any visible vortex. The stirring bars were Teflon coated. Data were plotted and analyzed with SigmaPlot or Kaleida-Graph.

## RESULTS

### Denaturation of LcA Induced by Mechanical Stirring

Agitation by stirring of 2.7 mL solution in all experiments was set at a speed that did not produce any vortex or visible turbulence at the surface, which was 13 mm above the “Star Head” magnetic stirrer (or 18 mm above the “Micro-Flea” stirrer bar) and 5 mm above the center of light path. The stirring therefore will be considered as mild. Figure 2 (curve 1) shows that mild stirring of LcA with the “Star Head” bar led to rapid loss of its CD signal related to  $\alpha$ -helical secondary structure as a function of time. An interruption of the agitation immediately stopped the loss of CD signal, but resumption of the agitation restored the time-dependent decline of the negative





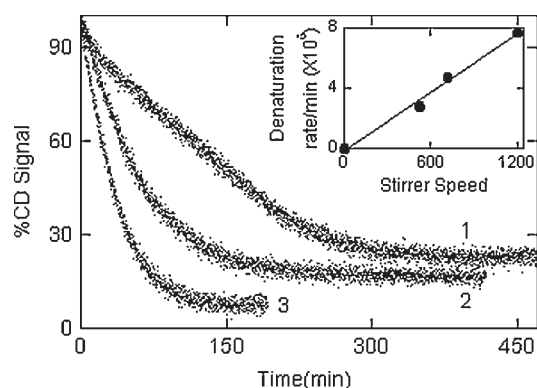
**Figure 2.** Effect of stirring a solution of LcA on the change in its ellipticity. A: 2.6 mL solution of LcA (20  $\mu$ g/mL 10 mM NaP, pH 7.4) was stirred at 1200 rpm at 20°C while its CD at 222 nm was continuously monitored. Stirring by the a “Star Head Stir Bar” (10 mm Dia  $\times$  8 mm H) at the bottom of the cuvette was 13 mm from the liquid surface and did not produce any vortex. Curve 1: Stirring of LcA was off or on at the indicated times. Curve 2: LcA in 5 mM DTT was held in an atmosphere of argon before and during continuous stirring. The difference in the initial values of ellipticity in curve 2 was due to the fact that stirring was initiated before recording of the CD. B: Far-UV CD spectra of LcA collected at 0 (open circle), 2 (closed circle), 10 (open triangle), and 90 (cross) min while being continuously stirred.

ellipticity. Same qualitative behavior was observed when a “Micro-Flea” stirrer bar was used for stirring (not shown). Since size and shape of stirrer bars can have significant effect on protein aggregation,<sup>26,27</sup> all subsequent experiments were conducted using the “Star Head” bar that occupies larger space in the cuvette allowing data collection on a smaller sample volume. The solutions at the end of the experiment were cloudy with fine white particles dispersed throughout the cuvette. At the initial phase of its appearance,

the white particles moved up and floated at the surface, but upon standing for an extended period or continuous stirring, they precipitated at the bottom, most likely by larger aggregation. We collected and washed the white precipitate by centrifugation. SDS-PAGE analysis (not shown) of the precipitate showed it to be the intact LcA as was the starting material. The precipitate however when incubated with the SNAP-25 substrate for an extended period at 37°C, failed to show any catalytic activity (not shown). To identify any secondary structural changes before aggregate formation, we also collected a CD spectrum of an LcA sample at various times during stirring (Fig. 2B). The negative ellipticity minima at 210 and 220 nm are generally attributed to helical structure.<sup>28</sup> In general, intensity of ellipticity at all wavelengths decreased with time of stirring but shape of the spectrum did not change significantly. The depressed signal intensity without changes in the spectral minima are consistent with loss of material and also absorption flattening.<sup>29</sup> The life of an LcA species having altered secondary structure presumably formed must be very short, because white precipitates start to appear soon after the start of stirring. Additionally, precipitation may result in spectral dominance of soluble monomers precluding characterization of any altered secondary structure.

In order to determine if the denaturation was due to increasing air oxidation (with residual oxygen in the nitrogen tank) by stirring, we carried out the experiment in 5 mM DTT in oxygen-free argon environment (Fig. 2A, curve 2). Denaturation remained unaffected by these reducing conditions. In another experiment, LcA was taken in a cuvette leaving no space between the liquid surface and the stopper. This too was ineffective in preventing the denaturation (data not shown). The results of these two experiments suggested that a probable cause of the denaturation was exposure of the LcA protein at the liquid–gas interface or liquid–solid interface mediated by mechanical agitation of stirring.

The rate of denaturation increased with increasing stirring speed (Fig. 3) such that a replot of the initial rates as a function of stirring speed was linear (Fig. 3, inset). The denaturation by stirring also increased with temperature (Fig. 4A). An activation energy of 75.6 kJ/mol was calculated for the denaturation reaction from an Arrhenius plot (Fig. 4B) of the data in Figure 4A.



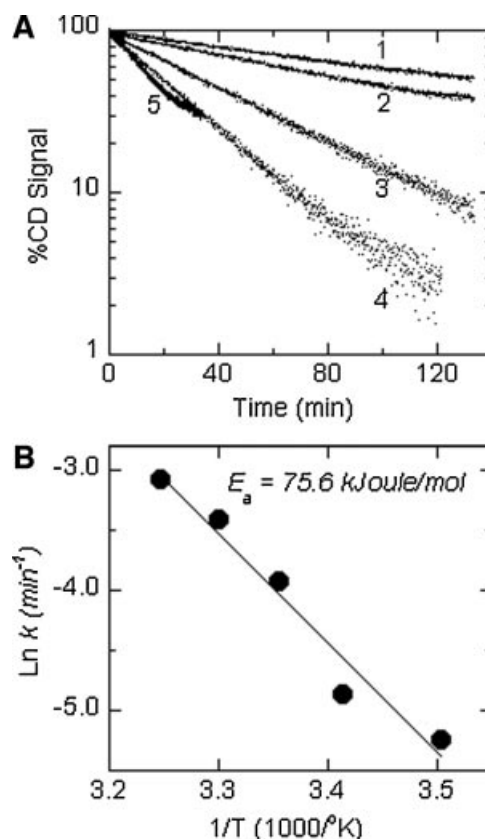
**Figure 3.** Effect of stirring speed on LcA denaturation monitored by following loss of the CD signal. Solutions of 2.6 mL of LcA (20  $\mu\text{g/mL}$  10 mM NaP, pH 7.4) was stirred at 520 (curve 1), 720 (curve 2) and 1200 rpm (curve 3) at 20°C while its CD at 222 nm was continuously monitored. Stirring by a “Star Head Stir Bar” (10 mm Dia  $\times$  8 mm H) at the bottom of the cuvette was 13 mm from the liquid surface and did not produce any vortex. The initial ellipticity of each solution ( $-12.5$  mDeg) was set at 100%. Inset, The apparent first-order rate constants were replotted as a function of stirring speed.

#### BSA and Other Standard Proteins are not Affected by Mechanical Stirring

Under identical experimental conditions of Figure 2, BSA and ovalbumin remained completely unaffected by stirring (Fig. 5). Thermolysin, a zinc endopeptidase and the closest structural-functional analogue of BoNT LCs, was also fully stable to mechanical stirring, as was carboxypeptidase B, another zinc-containing enzyme. However, the metalloproteins anthrax lethal factor and alcohol dehydrogenase were denatured by stirring but to significantly lower extents than LcA (Fig. 5, inset).

#### LC and Hc Domains of all BoNT Serotypes are Also Denatured by Stirring

When the LCs of other BoNT serotypes were subjected to mechanical stirring, all showed progressive loss of the CD signal (Tab. 1) eventually resulting in white precipitates. Compared with LcA, the rate of denaturation of LcB was much more pronounced. LcG on the other hand was most resistant to denaturation and precipitation among all LCs tested, although the effect of mechanical stirring was clearly visible.

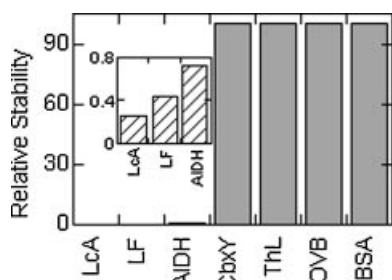


**Figure 4.** Effect of temperature (A) on LcA denaturation during stirring at 1200 rpm. A: Initial ellipticity values at 222 nm of each solution was normalized to 100%. LcA (20 mg/mL) was held at 10°C (curve 1), 20°C (curve 2), 25°C (curve 3), 30°C (curve 4), and 35°C (curve 5). Natural logarithms of first-order rate constants ( $k$ ) calculated from these curves are plotted against reciprocals of absolute temperature (K) in an Arrhenius plot in B.

Table 1 also compares the effect of mechanical stirring on BoNT Hc domains. Like LcA all Hc domains were also denatured to various extents, resulting in white precipitates. Although denaturation of HcA closely resembled that of LcA, no such generalizations could be made with these domains from serotypes B and E. HcC appeared to be most sensitive to stirring among all the Hc domains tested.

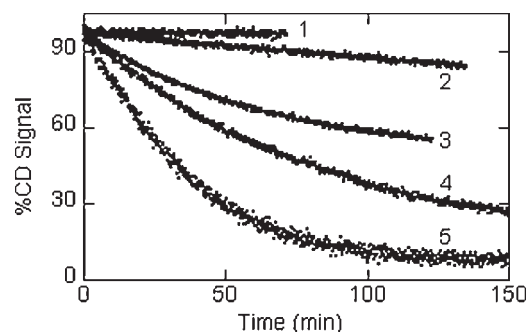
#### Nonionic Detergents Stabilize LcA from Denaturation by Stirring

In order to find a condition that could prevent the denaturation and precipitation of the LCs, we stirred LcA in the presence of various reagents



**Figure 5.** Effect of stirring on the stability of various proteins. Each protein (20  $\mu$ g/mL 10 mM NaP, pH 7.4) was continuously stirred at 20°C for at least 200 min while we monitored its CD ellipticity at 222 nm. Inverse of the rates of ellipticity change were calculated and are expressed as relative stability with the value for BSA set at 100%. For a better comparison, the values of the three least stable proteins are shown in the inset at an expanded scale. Abbreviations: BSA, bovine serum albumin; OVB, human ovalbumin; ThL, thermolysin; CbxY, carboxypeptidase B; AIDH, aldehyde dehydrogenase; and LF, anthrax lethal factor.

(Fig. 6). The nonionic detergent Tween-20 at a low concentration of 0.1% almost completely protected the LcA from denaturation. Triton X-100 was also significantly protective. Glycerol, a commonly used protein stabilizer, when used at a much higher concentration of 10%, had an insignificant effect on the stability of LcA by stirring (not shown). These results suggested that exposure of hydrophobic LcA residues by stirring may be prevented and protected by the detergents. BSA added at a high concentration of 5.5 mg/mL



**Figure 6.** Effects of protein stabilizers on stirring-induced denaturation of LcA. Surfactants and other reagents were added to LcA (20  $\mu$ g/mL) that were continuously stirred at 20°C while monitoring CD. Initial ellipticity values at 222 nm of each solution was normalized to 100%. Curve 1, 0.1% Tween-20 in 0.1 M NaP, pH 7.4; curve 2, 0.1% Triton X-100 in 0.1 M NaP, pH 7.4; curve 3, 5.5 mg/mL BSA in 0.1 M NaP, pH 7.4; curve 4, 0.1 M NaP, pH 7.4; curve 5, 10 mM NaP, pH 7.4.

afforded some protection from precipitation but was significantly much less effective than Tween-20 or Triton X-100.

## DISCUSSION

### Extreme Instability

Protein denaturation by shaking or mechanical agitation is an old observation. As early as 1927, Wu<sup>30</sup> (and the references therein) reported shaking a solution of protein destabilizes or denatures protein, forming aggregates and precipitates which are often catalytically inactive. Yet, destabilization leading to aggregation still remains a common problem with protein pharmaceuticals.<sup>31,32</sup> These destabilizations are usually attributed to exposure of kinetically stable unfolded<sup>33</sup> protein molecules to air–water or water–solid interfaces that enhance the aggregate formations.<sup>34</sup> Therefore, avoidance of unnecessary shaking or agitation has been a common practice when working with proteins. What makes the denaturation of BoNT LC domains remarkable is their extreme sensitivity to mechanical agitation when compared with such standard proteins as BSA and ovalbumin. The agitation conditions employed in this study was extremely gentle, avoiding (1) any visible vortex, (2) exposure to oxygen, (3) exposure to air or gas, and (4) extended time. Agitation-induced denaturation and precipitation of proteins observed recently

**Table 1.** Relative Stability of LC and Hc Domains of Various BoNT Serotypes Compared With BSA

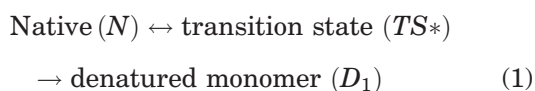
BoNT Domain	Relative Stability
LcA	0.25
LcB	0.11
LcD	0.31
LcE	0.21
LcG	2.50
BSA	100.00
HcA	0.26
HcB	1.04
HcC	0.19
HcE	0.42
HcF	0.47

Relative stability values were computed from the rate of loss of CD signal of each protein (20  $\mu$ g/mL) at 20°C as they were continuously stirred at 1200 rpm by a magnetic stirrer in experiments similar to that represented by curve 2 of Figure 2A. Reciprocal value of rate of loss of CD signal for BSA was taken as 100.

were done under a lot harsher conditions than in our studies. For example, albutropin precipitation was induced by shaking the protein at 600 rpm, allowing extensive bubble formation with air (oxidation) for 20–100 h at 25°C,<sup>35</sup> three different proteins were agitated at 400 rpm (with double air space suitable for oxidation by bubble formation) for 36–40 h or “high speed orbital mixing” on a vortex mixture for 30 min at 23°C,<sup>32</sup> and anti-venom proteins were vortexed “until completely foamed”.<sup>36</sup> Although our normally applied agitation speed of 1200 rpm is higher than some of the cited speeds, the rate of precipitation of LcA at 300 rpm (Fig. 3) was still at least 30-fold higher than that of BSA at 1200 rpm (Tab. 1). As shown in Figure 5 and Table 1, most of the BoNT LC and Hc domains were less than 1% as stable as BSA when subjected to identical agitation conditions. Being metalloproteins, these BoNT LCs were again extremely sensitive to mechanical agitation when compared with metalloenzyme, carboxypeptidase B. A protein that is most related to the BoNT LCs both structurally and functionally is thermolysin, a Zn-endopeptidase from *Bacillus thermoproteolyticus*. Unlike the BoNT LCs, however, this protein too remained unaffected by mechanical stirring. Hc domains from five BoNT serotypes examined were all 1% or less stable than BSA towards mechanical agitation (Tab. 1). These observations suggested that extreme instability of LCs to agitation may be a unique property among globular proteins but is a general property of BoNT domains.

### Mechanism of Aggregation

Many protein aggregation pathways have been analyzed by the well-known Lumry–Eyring framework,<sup>37</sup> represented in scheme I (Eqs. 1 and 2):



### Scheme I

The CD technique employed in these studies could not detect any intermediate such as  $TS^*$  or its aggregate-competent  $D_1$  (Fig. 2B) but agitation might have simply enhanced the formation of  $D_1$

that rapidly formed the inactive species  $D_m$  and  $D_{m+1}$ . In the absence of significant agitation, the catalytically inactive or poorly active  $D_1$  may have accumulated in a solution yielding suboptimal catalytic activity of a LC preparation. Indeed, large variations in the catalytic activity of the same LcA are reported.<sup>15–18</sup> Mild agitation only enhanced the formation of precipitates. Precipitation of LcA has also been documented under nonagitation conditions.<sup>22</sup>

Denaturation and aggregation of proteins is a complex process that is affected by temperature, pH, salt, protein concentration, shear force, viscosity, agitation, and additives.<sup>31,32</sup> Two dramatic effects on LC precipitation were increasing stirring speed and temperature, both increasing the precipitation (Figs. 3 and 4). Agitation-induced precipitation of insulin<sup>34</sup> and sickle cell oxyhemoglobin<sup>38–40</sup> are also affected by agitation speed and temperature. A linear Arrhenius plot (Fig. 4B) suggests a single common mechanism<sup>41</sup> is responsible for LcA precipitation over the temperatures (10–35°C) employed. The calculated  $E_a$  of 76.5 kJ/mol for LcA under stirring ( $N \rightarrow D_{m+1}$  Eq. 3 in Scheme I) is considerably lower than those for other proteins determined under stationary conditions ( $N \leftrightarrow TS^* \rightarrow D_1$  Eq. 1 in Scheme I): 222 kJ/mole for  $Ca^{++}$ -ATPase,<sup>41</sup> 205 kJ/mole for soluble RNase,<sup>42</sup> and 230–258 kJ/mole for subtilisin.<sup>43</sup> Although not determined under stationary condition for LcA, this large difference at least partly represents the contribution of stirring towards denaturation–precipitation of LcA.

Oxidation of amino acid residues is commonly encountered in protein denaturation. By conducting the experiments under reducing conditions of DTT, and under argon atmosphere (Fig. 2), we demonstrated that the agitation-induced precipitation of LcA was not due to oxidation of protein thiols in generating intermolecular disulfide bonds. Proteins are only marginally stable thermodynamically, and protein native conformation is flexible, having an ensemble of kinetically unstable sub-states including partially unfolded ones having contiguous surface hydrophobicity.<sup>31,33,44</sup> Our results suggest that LcA and other BoNT domains may have more such partially unfolded states than other proteins. Multiple catalytically active conformational states of LcA were recently demonstrated.<sup>20</sup> Mechanical agitation of LcA might expose these hydrophobic patches to the air–liquid interface, producing aggregation-competent intermediates that accelerate their aggregation. Because elimination of any air interface in



the stirring solution did not prevent LcA precipitation (see Results Section), we conclude that exposure of unfolded protein regions to the hydrophobic liquid–air or liquid–solid interface favored LcA precipitation as has been observed with insulin.<sup>34</sup> Stirring may have just accelerated the process. It is also possible that the BoNT domains were increasingly exposed to the hydrophobic Teflon surface of the magnets during stirring; similar effects of Teflon on insulin aggregation<sup>34</sup> and immunoglobulin aggregation<sup>45</sup> have been documented.

The amphiphilic nonionic surfactants are often used in preventing protein aggregation and unwanted adsorption, especially in working with membrane proteins. They provide sufficient hydrophobicity in protecting hydrophobic proteins from collapsing into precipitates in aqueous solutions. Including Tween-20 in the LcA stirring solution completely prevented LcA precipitation (Fig. 6). This result suggests LcA to be a hydrophobic or membrane protein. Yet, all the BoNT domains studied here cannot be categorized as hydrophobic proteins from their hydrophobicity index calculations.<sup>46,47</sup> It is thus likely that LcA (and other BoNT domains reported here) are kinetically unstable<sup>33,44</sup> with exposed hydrophobic patches. The nonionic detergent Tween-20 (and Triton X-100) protected these partially unfolded hydrophobic patches from becoming an aggregate-competent species and prevented aggregate formation. It is worth mentioning that BoNT toxicity persists in cultured neurons and human from days to more than a year.<sup>48–51</sup> Recent reports point to their membrane localization in the neurons<sup>52,53</sup> that provide an amphiphilic environment.

### Implication of Instability

What are the implications of the present findings? Protein aggregation is a common problem with protein pharmaceuticals.<sup>31,32</sup> Although protein denaturation by agitation is well known, extreme lability of the BoNT domains compared to such standard proteins as BSA, ovalbumin, and even the BoNT LC structural–functional analogue of thermolysin may suggest this is a unique feature of all BoNT proteins and domains. The whole 150-kDa BoNT/A toxin was reported to undergo rapid inactivation,<sup>54</sup> and to harness the full potential of the whole BoNT/A toxin as a therapeutic agent, a pharmaceutical composition was developed for

preventing its ease of denaturation.<sup>55</sup> Investigators working with BoNT domains have experienced variable results: from very poor to very high in protein yield (James J. Schmidt, personal communication), solubility,<sup>22</sup> stability,<sup>16,17,19</sup> catalytic activity Ahmed 2006, Unpublished],<sup>15–18</sup> and immunogenicity.<sup>21</sup> Such discrepancies may lead to nonreproducibility of published results (James J. Schmidt, personal communication), especially those directed towards therapeutic development. While reasons of the discrepant findings may be many, our results point to one probable common source: extreme lability of BoNT domains. This lability is enhanced by mild agitation that need not be deliberate or intentional, even slight swirling of a container of the protein(s) will increase the exposure of partially unfolded molecules to liquid–air and liquid–solid interface. In fact complete inactivation was observed in a stock solution that was repeatedly used (and got unintentionally mild agitation during handling) to take out LcA aliquots for activity measurements, and in tubes where microliter aliquots of LcA were allowed to climb down the wall of a reaction tube [Ahmed, 2001, unpublished]. Some investigators have added Tween-20 to overcome such problems.<sup>56</sup> Appreciation of this rather unusual property of BoNT domains may minimize some of the above problems. Our demonstration that physical and catalytic integrity of LcA can be maintained by adding Tween-20 and Triton X-100 should help to overcome many of the discrepant results obtained with this therapeutically important reagent.

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